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<b>(21) International Application Number:</b> PCT/US97/20989 <b>(22) International Filing Date:</b> 12 November 1997 (12.11.97) <b>(30) Priority Data:</b> 60/030,549 13 November 1996 (13.11.96) US <b>(71) Applicant (for all designated States except US):</b> QBI ENTERPRISES, LTD. [IL/IL]; P.O. Box 741, 74106 Nes-Ziona (IL). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DEISS, Louis, Paul [US/US]; 536 West Barry, Chicago, IL 60657 (US). YEHIELY, Fruma [US/US]; 536 West Barry, Chicago, IL 60657 (US). EFIMOVA, Elena [RU/US]; 720 S. Laffin, Chicago, IL 60607 (US). VASQUEZ-IASLOP, Nora Cecilia [MX/US]; 849 N. Mapleton, Oak Park, IL 60302 (US). EINAT, Paz [IL/IL]; Neve Nir 1/27, 74042 Nes Ziona (IL). <b>(74) Agents:</b> KOHN, Kenneth, I. et al.; Kohn & Associates, Suite 410, 30500 Northwestern Highway, Farmington Hills, MI 48334 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

**(54) Title:** GENE IDENTIFICATION METHOD**(57) Abstract**

A method for the identification of genes that are essential for the maintenance of specific cell phenotypes is disclosed. The method includes the initial step of identifying a cell type with a phenotype of interest. Gene inactivation is performed on an aliquot of cells of the cell type of interest. Positive selection is then performed to an aliquot of the cell culture to which gene inactivation has been applied. Cells which continue to maintain the phenotype following gene inactivation have not had the gene of interest inactivated whereas cells in which genes necessary for maintaining the phenotype have been inactivated have been lost. Utilizing subtraction analysis between treated and untreated aliquots the gene in the cells which has been inactivated that affects the phenotype of interest is identified. Genes that are identified by the method are also disclosed as well as antibodies directed against the gene product of the identified genes. Further a customized kit for the practice of the gene identification method is also disclosed.

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GENE IDENTIFICATION METHOD

## BACKGROUND OF THE INVENTION

## 5           1. FIELD OF THE INVENTION

The present invention relates to a method of identifying genes, specifically genes that maintain specific cell phenotypes.

## 10           2. DESCRIPTION OF RELATED ART

There are methods available to isolate and identify specific genes. However these methods are not efficient and rapid. Applicant has previously disclosed the Technical Knock Out (TKO) selection method which has the advantage of rapid isolation of genes that inhibit proliferation in a specified restrictive environment [Deiss and Kimchi, 1991; Deiss et al, 1995; Kissil et al, 1995; Deiss et al, 1996]. However, this method has the limitation of requiring a phenotype that can be efficiently selected against, such as a cell growth arrest or cell killing phenotype.

Recently Smith et al [1995] and United States Patent 5,612,180 have described a method called genetic footprinting to identify genes. The method involves mutagenesis of potentially large numbers of genes followed by a genetic selection of the cells containing the mutated genes. This is followed by retrospective analysis of the effect of individual gene inactivation on the behavior cells containing these inactivations. From this information new genes are determined. This method has significant disadvantages for large scale gene identification. The genetic footprinting method involves mutagenesis by gene insertion and because of this requires a haploid target which imposes a limitation on the method. Second, the method of determining the effect

of each gene inactivation on the fitness of the cells containing the mutation involves a PCR amplification of the target gene which requires prior knowledge of the nucleotide sequence of all the target genes that will be studied which limits the gene base which can be searched. It would be useful to have a method which does not require a haploid target and does not require a known sequence.

It would be useful to have a rapid method which can identify genes to be isolated that are essential for the maintenance of specific cell phenotypes where positive selection exists for the phenotypes. These identified genes are excellent targets for the development of pharmacological inhibitors which would also act clinically to inhibit the specific phenotype. In other words it would be useful to have a tool which can effectively identify pharmacological targets for inhibition of deleterious phenotypes.

## SUMMARY OF THE INVENTION

According to the present invention, a method for the identification of genes that are essential for the maintenance of specific cell phenotypes is disclosed. The method includes the initial step of identifying a cell type with a phenotype of interest. The method allows the phenotype of interest to be phenotypes relating to growth, phenotypes relating to release of factors and phenotypes relating to other basic cell functions.

Gene inactivation is performed on an aliquot of cells of the cell type of interest. Possible methods of gene inactivation include Genetic Suppressor Element (GSE) inactivation, Random Homozygous Knock-Out (RHKO) inactivation, or Technical Knock Out (TKO) inactivation.

Positive selection is then performed on an aliquot of the cell culture to which gene inactivation has been applied. The positive selection includes manipulations that test the ability of cells to survive under specific culture conditions, ability to express a specific factor, changes in cell structure, or differential gene expression.

Cells which continue to maintain the phenotype following gene inactivation have not had the gene of interest inactivated whereas cells in which genes necessary for maintaining the phenotype have been inactivated have been lost. Utilizing subtraction analysis between treated and untreated aliquots the gene in the cells which has been inactivated that affects the phenotype of interest is identified. The subtraction analysis can include the methods of differential display, representational differential analysis (RDA), suppressive subtraction hybridization (SSH), serial analysis of gene expression (SAGE), gene expression microarray (GEM), nucleic acid chip technology, or direct sequencing.

The invention further discloses the genes that are identified by the method of the present invention and for antibodies directed against the gene product of these identified genes. The present invention also provides for a customized kit to practice the method of the present invention.

#### DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a schematic representation of a general outline of the method of the present invention; and

FIGURE 2 is a schematic representation of the method of the present invention with a regulated anti-sense cDNA expression library.

#### DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, a method for the identification of which genes are essential for the maintenance of a specific cell phenotype is disclosed. Phenotypes that can be studied are those for which changes can be monitored in either haploid or diploid cells. The method requires two general steps. The first is the inactivation of genes in the cell by any method known in the art and then in the second applying positive selection for the phenotype of interest followed by the identification via a subtraction analysis of the gene in the cells which has been inactivated that affects the phenotype of interest. By this method, a collection of genes that are essential for the maintenance of a specific phenotype are identified at the conclusion of the procedure. The invention further discloses the genes that are identified by the method of the present invention and for antibodies directed against the gene product of these identified genes.

Briefly, the method includes initially the identification of a cell type for which genes controlling its phenotype are needed. Once the cell type has been identified, where required for the method an expression cDNA library is constructed of the cells as they are expressing the phenotype.

In general three methods are available for the gene inactivation step: Genetic Suppressor Element (GSE) [Holzmeyer et al, 1992; Roninson et al, 1995; Gudkov et

al, 1994], Random Homozygous Knock-Out (RHKO) [Li and Cohen, 1996], and Technical Knock Out (TKO) described herein above. Of these methods the TKO method is preferred as it adaptable to the second step of the present method as described herein below in Example 1. Any method for gene inactivation may be used with existing or later derived methods which can be adapted to work with the second step of the present method is preferred.

Following gene inactivation treatment, an aliquot of the treated cells are exposed to a positive selection. That is, the cells are exposed to conditions requiring/activating the phenotype of interest. A reserved aliquot of the treated cells is not exposed. Following positive selection cells which continue to express the desired phenotype remain and those cells which cannot maintain the phenotype are lost. The method then provides for determining the gene that was not expressed in the lost cells by a "subtraction" analysis by any method known in the art, generally utilizing a comparison between the reserved cell aliquot and the cells remaining after positive selection. It should be noted that many aliquots can be tested and screened. The gene(s) identified is at least one of the genes which controls the phenotype.

The relative abundance of the differences between the "targeted" and "untargeted" aliquots are simultaneously compared using a "subtraction" analysis (differential analysis) technique such as differential display, representational difference analysis (RDA), GEM-Gene Expression Microarrays (Schena et al., 1995; Aiello et al., 1994; Shen et al., 1995; Bauer et al., 1993; Liang and Pardee, 1992; 1995, Liang et al., 1993; Braun et al., 1995, Hubank and Schatz, 1994; United

States Patent Number 5,545,531), suppressive subtraction hybridization (SSH) and direct sequencing (WO96/17957).

In the preferred method the procedure involves the transfection of targets cells with an anti-sense expression library followed by the positive selection of cells which have maintained a specific phenotype in the face of a specific challenge to the phenotype. It should be noted that one construct can be tested or many can be tested simultaneously in this method including over 100,000 constructs from an expression library. Cells in which an anti-sense inactivation has targeted a "sense" gene essential for the selected phenotype will be lost during the selection. Applicants have found that in general one cell has incorporated only one construct.

In this embodiment the next steps are to identify and isolate anti-sense expression vectors that are lost from the cell population due to cell loss during positive selection, that is, that induce a disadvantage in transfected cells during specific, positive selection resulting in the loss of the cell carrying the vector. These vectors are identified by subtracting the anti-sense expression vectors present after the selection from those present before the selection utilizing the reserved cell aliquot. This difference represents the vectors that express anti-sense against gene(s) that are essential for the maintenance of the selected phenotype.

These vectors are then recloned and sequenced. The identified anti-sense expression vectors are re-tested individually for the ability to inactivate the specific phenotype. With the sequence identified, the sense gene controlling the phenotype of interest can be identified using standard methods known in the art.

More specifically, the first part of the method consists of transfecting a target cell culture aliquot with an anti-sense expression library. The library is



generated by cloning a cDNA library in the anti-sense orientation into an expression cassette that will express the anti-sense strand at a high efficiency. The cassette also contains a resistance marker that allows for selection of cells that have been successfully transfected. The cells that are transfected are ones that express a phenotype of interest.

The transfection results in a pool of cells that will express anti-sense messages against a large number of the genes expressed in the cell. These anti-sense messages will inactivate the functional expression of the corresponding sense message. This results in a pool of cells "knocked out" for the expression of many different genes. In many cases due to the vector system used, applicant have noted that the resulting cells will contain only a single anti-sense expressing vector.

When a transfected cell loses a specific phenotype, the anti-sense identity of the sense gene that has been knocked out is identified by isolating and sequencing the anti-sense expression cassette in the reserved unselected (untreated) aliquot. The anti-sense strand on the anti-sense expression cassette is the complement of the sense gene. If the anti-sense strand is not a full length anti-sense, or does not match a sequence of a known gene, then the gene fragment can be used as a hybridization probe in order to isolate the full length gene. In essence, the anti-sense expression vector serves as a tag to identify the gene inactivation event of interest.

The method involves the selection of the pool of anti-sense expressing cells for the specific phenotype. The goal of the selection is to separate the majority of cells which continue to maintain a specific phenotype from the rare cells in which an anti-sense inactivation event has specifically knocked-out a gene that is essential for the maintenance of the specific phenotype.

This can be based on virtually any kind of positive selection means. These selection means can be varied as is known to those skilled in the art. However, the following is a non-exhaustive list and is not to be construed as limiting the present invention to these listed means.

The selection means is based on the ability of the cells to:

1. Grow or survive under specific culture conditions, that is the actual selection is for the growth or survival of the cells. In an embodiment, this can be basic culture conditions, such that the selection is for growth or survival-essential genes. The selection conditions could include sub-effective doses of specific factors which at effective doses would cause growth arrest or cell killing. In this case the selection is for the identification of knock-outs which sensitize the cells to the specific added factor.

In another embodiment, the selection can be in combination with a factor that normally does not cause an arrest or killing function. In this case a knock-out could be selected which only in combination with the added factor are effective in arresting or killing cells.

In a further embodiment, the selection can be for the inability to grow or survive when a parasite or infectious agent is added to the cell of interest. In this case the selection would be for knock-outs that are targeting genes that are specifically essential for some aspect of viral or parasitic function within a cell that are only essential when that cell is infected. Since some viral infection result in the induction of survival factors (such as CrmA, p35) it is likely that at least some cell functions are different and potentially selectively needed during viral, parasite growth.

2. The second type of selection means is for the expression of a specific factor that can be measured and this measurement can be adapted for a selection. This factor can be anything that is accessible to measurement, including but not limited to, secreted molecules, cell surface molecules, soluble and insoluble molecules, binding activities, activities that induce activities on other cells or induce other organic or inorganic chemical reactions.

3. The third type of selection means is for changes in cell structure that are detected by any means that could be adapted for a selection scheme. This includes, but is not limited to, morphological changes that are measured by physical methods such as differential sedimentation, differential light scattering, differential buoyant density, differential cell volume selected by sieving.

4. The fourth type of selection means is based on differences in gene expression that can be directly measured. This includes changes in cell surface markers, changes in biochemical activities, any changes that would be re-selected in changes in binding of fluorescent labeled probes that could be used in conjunction with a Fluorescence Activated Cell Sorter (FACS) or any property that can be used as a basis for a selection.

5. The fifth type of selection means is based on differences in gene expression that can be indirectly measured. This includes changes in transcription factor activity that are measured by a synthetic gene construct encoding a selective marker (such as a drug resistance marker or a cell surface marker that could be used in a FACS selection). This category would also include changes in mRNA stability, mRNA localization, mRNA translation control. All of these changes could be the basis of a selection because a synthetic construct which

is controlled by one of these regulatory events could be constructed which would drive the expression of an easily selected gene product.

5 The third part of the method involves steps  
identifying the anti-sense knock-outs that specifically  
inhibit the phenotype of interest. Since the selection  
of the anti-sense transfected cells is based on the  
maintenance of the phenotype of interest, the cells of  
interest (those loosing the phenotype) will not be  
10 present after the selection but will be present before  
the selection. Since the functional changes are caused  
by expression from anti-sense expression vectors and the  
inactivated genes can be identified by sequence analysis  
of the cloned anti-sense cDNA insert, the goal of this  
15 step is actually to identify the anti-sense expression  
vectors that are lost from the population of cells during  
the selection procedure.

The anti-sense inserts are cloned into a defined  
position on the vector and the sequence elements  
20 surrounding the site are known, so all the cDNA inserts  
can be amplified with the use of a PCR amplification  
using primers from the sequences that surround the insert  
site. Thus the goal becomes to identify DNA molecules  
present in one population and not in another. This is  
25 accomplished by a variety of subtraction techniques.  
Some of the methods that will be used are summarized  
below as is known to those skilled in the art. However,  
the following is a non-exhaustive list and is not to be  
construed as limiting the present invention to these  
30 listed means. Various differential hybridization methods  
as well as different subtractive hybridization techniques  
will be used. They are summarized in some detail in the  
methods section.

Once fragments are identified that are lost during  
35 the selection and are candidates for genes of interest

their function must be confirmed and the gene identified in the fourth part of the method. The fragments will be recloned into the anti-sense expression cassette and individually re-transfected into the target cell to  
5 determine whether the expression of the isolated fragment can really change phenotype. If the phenotype is really lost as is predicted then the isolated fragment will be sequenced and used to isolate the full length sense gene. It will also be determined whether the fragment is indeed  
10 anti-sense with the use of strand specific probes. The sense gene fragment will be used to derive antibodies that can be used to monitor expression levels to determine if there has been a functional anti-sense knock-out [Deiss et al., 1995].

15 The present invention is a genetic method for identifying genes that are essential for the maintenance of specific cell phenotypes. The method requires that the specific phenotype can be positively selected. These identified genes are excellent targets for the  
20 development of pharmacological inhibitors which would also act clinically to inhibit the specific phenotype. Thus the present invention provides a gene discovery tool which can effectively identify pharmacological targets for inhibition of deleterious phenotypes.

25 The following are several examples but this list is not to be construed as limiting the present invention to these listed examples.

**Phenotypes related to growth or survival**

Addressing the problem of unusual growth: This  
30 includes the problem of cancer but is not limited to cancer but is applicable to all aberrant growth events. The method of the present invention can be used to identify genes that are essential for the growth of cells transformed under general or specific conditions.

To define genes that are essential specifically for transformed cells, an anti-sense cDNA library would be introduced into a transformed and the non-transformed cells that it was derived from. The anti-sense constructs that interfere with transformed cell growth and not from the non-transformed cells are found by subtracting the anti-sense RNA molecules expressed in surviving cells from both transfections. Knock-outs specifically absent in the transformed cells but present in the non-transformed cells are desired. These are isolated by the methods described herein. The selection can be a most specific selection such as one where sub-lethal doses of chemotherapeutics are added during the selection. In this case the selection would include gene knock-outs that sensitize the cells to chemotherapeutic treatments.

The factors added during the selection could be ones that are thought to be present at the site of tumors. Thus the selection would include events that sensitize cells to a localized tumor effect and could increase the specificity of anti-cancer treatment. Any growth or survival event could be used as a basis not just cancer related.

The growth or survival phenotype can also be used as a way of eliminating populations of cells that are not necessarily growing improperly but which function in a manner that is deleterious. Thus virally infected cells or parasite harboring cells could be used as a target and the un-infected or non-parasite containing cells used to subtract. This would define all the genes that are specifically essential for the cell in the presence of these insults. These would of course be excellent targets for inhibiting viral or parasite spread.

**Phenotypes related to the release of factors:**

This class of selections includes events that increase or decrease the production of secreted factors. These include inflammatory mediators whose release could be modulated. For example, if the production of a specific mediator is necessary for normal immune function but is produced at lethal levels in aberrant situations (such as septic shock), then one could use the production as a screen and look for events that knock-out or down-regulate productions. In a further embodiment, the selection can be done in the presence of sub-optimal doses of other drugs in order to identify sensitization events.

**Phenotypes related to changes in cell functions:**

These selection events are designed to identify genes that are essential for many basic cell functions that depend on any changes that can be externally selected.

Several permutations of the method of the present invention are possible and are presented as schematic diagrams in Figures 1-2. Figure 1 provides a general outline of the gene identification method of the present invention. In this version a population of cells is first transfected with an anti-sense cDNA expression library. The expression library in this scheme codes for a drug resistance marker that is used to select transfected cells. This results in a population of cells (Population 1) that all contain anti-sense expression cassettes. The population of transfected cells is then placed under a selection pressure. Cells that survive this selection constitute population 2.

Transfected cells that become sensitive to the selection procedure will be lost or at least reduced in abundance in population 2. In order to identify the

constructs that induce this sensitization the following procedure is performed. The expression cassettes contained in the two population are extracted from the cells. The cDNA inserts are excised by PCR amplification using primers that flank the cDNA cloning sites. This results in two pools of PCR fragments. To identify the elements that are lost during the selection a subtraction is done between the two pools. Elements are identified that are present in population 1 and absent or reduced in abundance in population 2.

To confirm that the subtracted fragments do indeed induce a sensitization to the selection procedure, individual fragments are recloned into the identical vector and than individually retransfected into cells. These cells are then individually assayed for sensitivity to the selection procedure. A correctly cloned element will induce sensitization of the transfected clones to the selection procedure.

Figure 2 provides a diagram of the method with a regulated anti-sense cDNA expression library. In this simple variation the object is to clone the anti-sense cDNA library into a vector in which expression of the anti-sense is regulatable. The method is then modified so that during the original transfection, the expression of anti-sense is turned "OFF". After cells are selected for the presence of the vector an aliquot of cells is harvested and vectors are extracted and inserts excised by PCR. This constitutes pool 1. The remaining transfected cells are treated to turn "ON" the expression of the anti-sense expression. An aliquot of these cells are taken after several cell divisions (pool 2). Again the aliquot of cells are extracted and cDNA inserts excised by PCR. Finally an aliquot of the cells with anti-sense turned "ON" is placed under a specific selection and cells after this selection are harvested.



Again following extraction and PCR amplification we have pool 3.

In this case we can perform two kinds of subtractions. The first subtraction would be pool 2 from pool 1. This identifies anti-sense inactivations that are lethal or growth arresting. The second subtraction, would be subtracting pool 3 from pool 2. This will identify anti-sense knock-outs which sensitize cells to the specific selection.

Additional permutations/variations on the method of the present invention can be made. The method can be used to identify different gene expression backgrounds. In this variation anti-sense induced sensitization in cells that express different genes is investigated. This can be accomplished by transfecting into cells that contain an inducible gene expression cassette. This cassette affords inducible expression of a specific gene construct we will call gene X for this example. Following transfection and selection for the presence of an anti-sense cDNA library and aliquot of cells is harvested, vector extracted and cDNA inserts excised by PCR. This is pool 1. The remaining cells are induced to express gene X. Allowing some time for expression, the cells are harvested, vectors extracted and cDNA inserts excised by PCR. This generates pool 2. The subtraction of pool 2 from pool 1 yields inserts that specifically sensitize cells to the expression of gene X.

In another variation the method of the present invention is used with different cell types. This variation involves transfecting two different cell types. This could be cells of different genetic background or of different tissue origins, or even from different organisms. In the simple diagramed case two cell types are transfected with the same anti-sense cDNA expression library. The different cell types are propagated in

different containers. Transfected cells are then selected for the presence of the library. The cells containing the library are harvested, vectors extracted and cDNA inserts are excised by PCR. For each cell type a different pool is generated. The subtraction between these pools, both pool 1 from 2 and pool 2 from 1 identify anti sense knockouts that are specifically lethal or growth arresting to one cell type but not the other.

In a further variation, the method of the present invention is used for determining the fitness of specific genes in a population. In all the versions described above, populations of PCR fragments are generated which potentially differ by some number of elements due to the biological activity of those elements. The subtraction of these pools is then used as a method to identify cDNA fragments which have biological effects when expressed. It is also possible to use the same pools to determine whether an anti-sense construct directed against a specific gene could confer some biological effect during some sort of selection. Specifically in this simple example, when two pools 1 and 2 have been generated for the operation such as in the examples, there are a variety of tools available to individually measure the relative abundance of anti sense construct representing specific cDNAs in pool 1 and pool 2. A variety of methods are known for quantitating the abundance of DNA molecules in different samples. Following is a non-exhaustive list: Southern blot analysis either using a fragment of the gene of interest as a probe against the two pools; Quantitative PCR with specific primers identifying the gene of interest; GEM analysis, using the pools 1 and 2 as the probes and hybridizing against chips of arrayed known genes. If the abundance of the anti-sense construct significantly decreases after a selection

then it is likely that anti-sense has sensitized the cells to that selection.

As shown in Example 2 and Table 2 herein below, sequences of genes have been identified by the method of the present invention (SEQ ID Nos:15-36). An antisense construct of these sequences delivered to a cell reduces a gene product (gene inactivation) and thereby provides sensitization of the cells to anti-Fas antibodies. In a preferred embodiment the sequences are SEQ ID Nos:19,20,23,25,26,36. These antisense constructs can be used therapeutically to sensitize the cells for antibody therapy. Antisense therapeutic construct can be delivered to the cells and can be rendered nuclease resistant as is known in the art [Agrawal, 1996; Calabretta, et al, 1996; Crooke, 1995; Felgner, 1997; Gewirtz, 1993; Hanania, et al 1995; Lefebvre-d'Hellencourt et al, 1995; Lev-Lehman et al., 1997; Loke et al, 1989; Wagner et al., 1996; Wagner, 1994; Radhakrishnan et al., 1990.]

The present invention also discloses novel gene sequences as set forth in SEQ ID Nos:18,21,25 and 30-32.

The present invention also provides for a customized kit to practice the method of the present invention. The kit would be assembled to include at least an expression cDNA library constructed for specified cells as they are expressing the phenotype. Further a culture of cells of the requested phenotype could also be provided in the kit.

The above discussion provides a factual basis for the method of identifying genes that are essential for the maintenance of specific cell phenotypes. The methods used are shown below and can be shown by the following non-limiting examples and accompanying figures.

## EXAMPLES

GENERAL METHODS:

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989).

Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). Reactions and manipulations involving other nucleic acid techniques, unless stated otherwise, were performed as generally described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference.

Recombinant Protein Purification is undertaken as generally set forth in Marshak et al, "Strategies for Protein Purification and Characterization. A laboratory course manual." CSHL Press, 1996 unless otherwise specified.

Vectors are constructed containing the cDNA of the present invention by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences (see below in specific methods for a more detailed description). Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such

as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors are introduced into cells or tissues by any one of a variety of known methods within the art (calcium phosphate transfection; electroporation; lipofection; protoplast fusion; polybrene transfection). The host cell can be any eucaryotic and procaryotic cells, which can be transformed with the vector and which will support the production of the enzyme. Methods for transformation can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995) and Gilboa, et al. (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see United States patent 4,866,042 for vectors involving the central nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods.

**General methods in immunology:** Standard methods in immunology known in the art and not specifically described were generally followed as in Stites et al. (eds), *Basic and Clinical Immunology* (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), *Selected Methods in Cellular Immunology*, W.H. Freeman and Co., New York (1980).

Immunoassays: In general, ELISAs are the preferred immunoassays employed to assess a specimen. ELISA assays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate other immunoassays, such as radioimmunoassays (RIA) can be used as are known to those in the art. Available immunoassays are extensively described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989.

#### Polyclonal and Monoclonal Antibody Production

**Antibody Production**: Antibodies may be either monoclonal or polyclonal. Conveniently, the antibodies may be prepared against a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the protein or peptide, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the protein are collected from the sera.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the protein or peptide fragment, generally a mouse, and isolation of splenic antibody producing cells. These  
5 cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

10 The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe,  
15 *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane  
20 *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992) The detectable moieties contemplated with  
25 the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase,  $\beta$ -galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium,  $^{14}\text{C}$  and iodination.

#### SPECIFIC METHODS

##### 30 Construction of anti-sense expression vector:

This method is not limited to any specific vector system. The actual requirements are that the vector system express at high levels anti-sense molecules and that they can be identified. In a preferred embodiment

the Epstein Barr Virus (EBV) Episomal vector is used [Deiss et al, 1991].

5 The EBV episomal vector consists of DNA segments that are necessary for the episomal maintenance of the episome both in bacteria (*E. coli*) and in human cells (this include an origin of replication and a trans-acting factor (EBNA-1). The episome also includes genes encoding resistance markers for selection either in bacteria or in human cells. Finally the vector contains  
10 a transcription cassette. Initially it will be based on the vector as described in Deiss et al. [1991], but the present invention contemplates any transcription cassette that produces high levels of anti-sense expression. The EBV episomal vector contains a RNA Polymerase II promoter  
15 and or enhancer driving the transcription of a synthetic transcript containing a set of cloning sites, a splice donor and acceptor site and a polyadenylation signal, followed by a second set of enhancers. This vector can be efficiently shuttled from animal cells to bacteria and  
20 vice versa. One procedure that allows for rapid shuttling is using the method of Hirt [1967] to extract episomal vectors from animal cells and using this preparation to transform *E.coli*. Applicants have observed that, on average, cells transfected with a  
25 library cloned into the vector contain only one expressing vector.

The specific choice of promoters and enhancers are dependent on the exact selection condition and the cell line used. This must be empirically determined for each  
30 selection condition as is known to those skilled in the art.

In an embodiment, the EBV vector can also contain an inducible expressed promoter such that the expression of the anti-sense library would be inducibly expressed by a



specific inducer. This will allow additional flexibility in designing selection protocols.

Construction of Anti-sense cDNA library

5 There are several methods available to construct directional cDNA libraries. Any of these methods would be sufficient since they result in the production of a directionally identified cDNA library and the practitioner can use the method they are most familiar with. The directional cDNA is then cloned into the  
10 expression cassette in the anti-sense orientation. A method that may be used is detailed in Deiss et al, 1991. Briefly, it consists of making cDNA by the method of Gubler and Hoffman [1983] and making the cDNA directional by the method of Meisner et al. [1987].

15 The mRNA is extracted from cells that have been cultured under a variety of conditions that mimic the actual selection conditions. This is designed to ensure that the library will include all the messages that are expressed in the target cell under selection conditions.  
20 RNA is prepared at time points that would contain messages that are always present as well as messages that are induced by the selection procedure. This is achieved by extracting RNA previous to the selection and at times during the selection. The various pools of RNA are then  
25 mixed together so that all possible RNA molecules are present [Deiss and Kimchi, 1991].

An alternative method that can be used consists of deriving a library of genomic DNA fragments cloned into the expression cassette. Since all the transcribed  
30 messages are derived from genomic DNA (with the exception of RNA edited messages; this will actually include mitochondrial DNA as well) this method would generate all possible messages. The directionality would be lost so the library would be only half anti-sense. Since the  
35 sense fragments are unlikely to frequently encode full

length proteins or have biological activity the anti-sense fragments would still likely produce the most frequent biological effects. The genomic fragments are produced by restriction enzyme cleavage of genomic DNA.

5 Only one library per species would be necessary to produce, since with the exception of the B and T cell receptors, genomic DNA does not differ in different cell types at least in mammals (again erythrocytes or any cells that lack nuclei are an exception).

10 In the case of the genomic library it will be necessary to determine whether any expressed fragments express a sense or an anti-sense message. This is done by using the insert as a strand specific probe both from the expressed and non-expressed strand in a Northern  
15 analysis. This indicates if the expressed fragment is sense or anti-sense in relation to the endogenously expressed gene.

Some sequence will match, some will not match, genes already deposited in the various databases. In the case  
20 where the identified gene matches the sequence of a gene already in a database this information would then enable the determination if the insert is a sense or anti-sense insert.

Transfection of the anti-sense library.

25 There are a large variety of methods to transfect DNA into cell lines and cell cultures. The most efficient method for each selection will be determined empirically based on experience and the known relative efficiency of each method.

30 The method selected must both efficiently delivery DNA into cells and not effect the biological responses that will be selected following the transfection. Viral vector system can also be used and this would entail producing infectious virus and infecting the target  
35 cells. Applicant has found electroporation to be an

efficient method, but other methods can be used as are known in the art.

Identification of differentially expressed antisense messages

5           The methods of identifying the differentially expressed antisense messages in a preferred embodiment will include the methods described in Braun [1996] and as described in Diatchenko et al. [1996]. These methods include a PCR amplification of subtracted populations.  
10   Appropriate restriction sites are included in the expression vector so that following PCR amplification of the cDNA inserts, the inserts are flanked by appropriate restriction sites. Restriction digestion is then used to produce templates that are useful for these techniques.

15           Another method that is used is the "GEM" gene expression microarray as described in Schena et al. [1995]. In this technique, PCR fragments corresponding to a set of specific plasmids (in this case antisense cDNA inserts contained in antisense expressing vectors or  
20   other DNAs as appropriate) are fixed to a glass template and this is hybridized with two fluorescently labeled probes. In this specific case, the probes are reverse transcribed antisense transcripts derived from cells transfected with the antisense expression library either  
25   before or following a selection.

Generation of efficient antisense inhibitor

          In addition to assaying expression cassettes where all the transcripts are directionally cloned in the antisense orientation, another strategy employed in the  
30   present invention is to generate randomly primed cDNA and cleave the cDNA with two restriction enzymes X and Y and clone the resulting mixture into two different expression cassette. In the first cassette site, X, would transcriptionally precede Y and the second cassette site,  
35   Y, would transcriptionally precede X. In this

arrangement, the cDNA is divided into sections that may have different abilities to serve as an efficient antisense inhibitor. The strongest differential signal is likely to be produced by the fragment that is the most efficient antisense inhibitor. Thus, the screening is more likely to produce a meaningful differential signal.

### SPECIFIC EXAMPLES

#### DETERMINATION OF COMBINATION OF METHODS

10

#### FOR PREFERRED EMBODIMENT

The method requires two distinct major steps as described herein above. It is greatly advantageous if this method can be applied to a wide variety of cells. It is therefore useful if both steps could be applied to a wide variety of situations.

15

In the first step genes are inactivated in order to determine whether individual genes are essential for a specific phenotypic change. It is advantageous if these inactivation will have a phenotype both in haploid cells and in diploid cells. Since many cells of interest are diploid in nature. Furthermore, it is also an advantage if the inactivation method allows for the rapid identification of the inactivated genes. This can be achieved in a variety of manners. The inactivation methods are generally based on one of three different principles.

20

25

The first principle is that genes can be functionally inactivated by expressing mRNA that is derived from the anti-sense strand of the sense message. This allows for inactivating the mRNA in the cell and does not require a specific gene dose. This can work for single copy or multiple copy genes either from haploid or diploid organism. It has been shown that anti-sense inactivation can be effective in a wide range of organisms including bacterial, plant and animal.

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35

Applicants and others have extended the original observation by generating anti-sense expressing cDNA libraries. Applicants termed this method Technical Knock Out (TKO). These libraries contain collections of many  
5 (usually 100,000 to 1,000,000) different anti-sense expression constructs that will individually express a single anti-sense RNA molecule when transfected into appropriate target cells. Since these libraries contain large collections of these vectors they in effect can  
10 express anti-sense RNA to virtually all expressed mRNA molecules. Several investigators have used these type of libraries to inactivate genes and change the phenotype of cells. Once an altered cell is identified the expression cassette contained in the cells can be  
15 identified since the expression cassette DNA sequence is known. Subsequently, the anti-sense expressed cDNA molecule that is contained in the expression cassette is identified. This can be achieved by a variety of methods; applicants have used two methods. The first  
20 method involves shuttling the vector from animal cells into bacterial cells. Once the vector is in bacterial cells it is easy to produce large amounts of the vector for further analysis. The second method we have employed involves PCR amplification of the cDNA inserts by  
25 designing PCR primers that flank the cDNA cloning site on the vector. The flanking vector sequences are known so it is easy to chose appropriate primers. PCR amplification with these primers amplifies any cDNA molecules that were present between the two primers. The  
30 anti-sense approach also allows for tagging of the inactivation event. That is the identity of the sense message generally can be determined by sequencing the anti sense construct. This construct can then be identified and isolated from the phenotypically altered  
35 cells.

The second gene inactivation method that fits these requirement is an inactivation method that relies on production of "dominant negative" fragments of genes from an expression cDNA library. This method is called the Genetic Suppressor Element method (GSE). It is based on the observation that small fragments of a gene when expressed may interfere with the normal function of the full length gene product and in fact interfere with the normal function. In this manner these gene fragments were called "dominant negatives". A GSE library thus consists of fragmented cDNA molecules which are cloned into an expression cassette. When expressed from translation initiation signals in the cDNA molecule or from translation initiation signals present in the expression cassette these gene fragments can interfere with gene function. In addition the libraries used in the GSE method also include some anti-sense fragments and therefore gene inactivation can occur either by anti-sense or by dominant negative inactivation of gene function.

The third method of gene inactivation that could be used is called "Random Homozygous Knock-Out (RHKO)". In this method gene inactivation is achieved in two steps. A retroviral vector is used to infect target cells. The integration of the retroviral element itself can lead to inactivation of one copy of a gene if the integration event itself functionally disrupts the normal transcription or activity of the gene in which it integrates. The retroviral vector used has an additional property that it encodes a transcription element that should transcribe into the chromosomal location in which it has integrated. In the case that this generates an anti-sense RNA transcript, additional copies of the gene could be inactivated. Thus this method also relies on anti-sense inactivation.

The TKO method was chosen for generating inactivations in these examples as the preferred embodiment because the other methods described above are not as compatible with the second step in the method of the present invention as the TKO procedure. However, as improvements become available in these methods they could be used.

In the GSE method both sense and anti-sense gene fragments are generated which are expected to have different biological activities. It is difficult to distinguish closely related gene fragments of this sort by the methods that will be used in the second part of the gene identification method of the present invention. Thus the rare molecules that cause biological changes when expressed would be very difficult to distinguish from many similar molecules that would not have biological effects. The molecules that do not have effects would in essence mask the active molecules.

The RHKO method was also difficult to adapt to a high throughput subtractive procedure. The potential anti-sense fragments generated in this procedure must be cloned out individually and this is a process that is hard to adapt to subtraction.

The TKO method was easily adapted to subtraction. The cDNA inserts contained in expression vectors should be all or at least mostly anti-sense in nature. The cloning procedure outlined in Deiss and Kimchi was used. This generates anti-sense cDNA libraries and results in libraries that are biased to be anti-sense. It is possible to obtain some sense cDNA inserts with this method. Thus since most of the fragments are antisense the subtraction step will be mainly between different cDNA fragments that were expressed as anti-sense constructs. Again the principle of the present invention is that the abundance of an anti-sense construct(s) that

induces a disadvantageous phenotype will be reduced after a biological selection. We wish to identify these constructs. Thus the TKO method was the method of choice for the gene inactivation step of the present invention. It can be used in a variety of cell populations, both in haploid, diploid and aneuploid cells. It can be easily scaled up to involve 100,000 events or more without undue expense. And it can be easily adapted to the subtractive methods that are needed in the second part of the method of the present invention.

The second step in the gene identification method requires that we can identify the loss of specific anti-sense gene constructs from a large population of anti-sense constructs that are not lost. This can be accomplished in a variety of different ways. Because it is a great advantage to be able to identify specific losses in the presence of large numbers of molecules that are not lost we needed a method that has a high throughput capacity. One method that fits this requirement involves using high density arrayed chips such as the GEM chips. These are arrayed dots containing specific DNA molecules corresponding to genes. The dots are arrayed at high density on a glass coverslip with the position of each dot and the identity of the DNA molecule fixed on each dot precisely determined. Two probes derived from different population of DNA or RNA molecules are labeled with two different fluorescent dyes and hybridized to the arrays. After appropriate washing the relative binding of the dyes at each dot is determined. The amount of dye bound at each spot reflects the abundance of the gene fixed on that dot relative in the whole population. Thus when two populations of DNA molecules are labeled with different dyes one can accurately determine whether there has been a change in relative abundance of individual molecules in the



population. If there is no change the ratio of the two dyes will be one. If there has been a change in abundance then the ratio of the two different dyes will also change. This method can rapidly measure the changes of large numbers of genes in a large population. A copy of the DNA fix on each dot is stored and can be retrieved for further analysis. Although in the following example we did not use the GEM method to measure the loss of anti-sense constructs it is a method that can be used in the practice of the present invention.

A second method uses to identify the loss of specific anti-sense gene constructs from a large population of anti-sense constructs that are not lost is called "Subtraction". This involves manipulating two populations of DNA or RNA molecules so that only molecules that are present in one population and not in another are recovered. The version that we actually used is called PCR-Select which is a commercially available kit from CLONTECH. Briefly

1. Two populations of double stranded DNA are generated. It is assumed that some of the dsDNA molecules are present in only one of the populations (or at least much more abundant in one population).

2. These populations are separately processed. The population that is assumed to have extra species of molecules is called the tester sample. The second population that is assumed not to have these specific species is called the driver. The tester population is separately ligated to two different linkers. This generates tester population 1 and tester population 2. The driver is left without linkers.

3. A series of manipulations including denaturation and renaturation of the driver and tester in various combinations is used. This results in generating a series of DNA molecules that have different set of

linkers at their ends. The only product that can be effectively PCR amplified at the end of the manipulations are those that are present in the tester and absent (or reduced) in the driver. These molecules are easily  
5 isolated after the last PCR step.

The net result of this method is that a population of gene fragments that are present in one population and lost or absent in another population is rapidly isolated. This is exactly what is needed in the gene identification  
10 method. These PCR products can be isolated by standard techniques and be used for further analysis as shown in Example 2.

#### EXAMPLE 2

##### 15 IDENTIFICATION OF GENES IN HeLa CELLS THAT ARE INVOLVED IN *fas* ANTIBODY SENSITIVITY

The method of the present invention was applied to HeLa cells treated with anti-Fas antibody in order to  
20 identify genes that when knocked-out cause sensitization of HeLa cells to the action of anti-Fas antibodies.

HeLa cells are derived from a human cervical carcinoma and were used in the original TKO [Deiss and Kimchi, 1991]. HeLa cells were used as an exemplar of  
25 the method of the present system as they are easily grown in culture, are easily transfected and respond to anti-Fas antibody treatment.

Anti-Fas antibody (Kamiya Biomedical Company, Seattle, Washington, catalog number: MC-060) is directed  
30 against Fas/CD95/Apo-1, a transmembrane receptor that is known to signal a death response in a variety of cell types. This antibody is an activating antibody, that is, the binding of the antibody mimics the effects of binding of ligand. Applying the appropriate dose to responding  
35 cells has been shown to lead to induction of cells death

(Deiss et al., 1996). HeLa cells respond to this treatment.

In this exemplar genes are identified that regulate the sensitivity of HeLa cells to killing by anti-Fas antibody. Specifically, genes are identified whose loss sensitizes HeLa cells to anti-Fas treatment.

The outline of the procedure is as follows:

1. HeLa cells were transfected with an anti-sense cDNA library.

2. Cells containing anti-sense expression vectors were isolated by selection with Hygromycin. Since the vector contains the Hygromycin resistance marker, the selection of the transfected cultures with Hygromycin generated a population of cells which contain the anti-sense expression cassettes.

3. Aliquots of this pool of cells were treated with anti-Fas antibody under two different experimental conditions. It should be noted that more conditions could be screened at the same time.

a. Treatment with a sub-lethal dose of anti-Fas antibody (10 ng/ml). Cells that are super-sensitive to treatment with anti-Fas antibody were killed whereas the majority of the population which is resistant to the treatment proliferated.

b. In the second condition, the cells were treated with a lethal dose of anti-Fas antibody (100 ng/ml). The cells were harvested at 24 hours, before the majority of cells had been killed. In this case, applicants were looking for anti-sense events that accelerate the killing associated with anti-Fas treatment as another type of sensitization.

4. Aliquots of the cells just before the treatment with anti-Fas antibody and just after the treatment with anti-Fas antibody were harvested. The DNA contained in each cell population was extracted.

5. The anti-sense cDNA inserts contained in these DNA samples were preferentially amplified through the use of PCR (see details below).

6. The pools of anti-sense cDNA fragments that were derived from cells after treatment were subtracted from those before treatment (see details below). This generated a set of cDNA fragments that were present in cells before treatment but were absent after treatment. These fragments are good candidates for sensitizing cDNA fragments. In other words, it is likely that expression of some of these fragments leads to the inactivation of genes which causes cells to become super-sensitive to anti-Fas antibody treatment. These super-sensitive cells are quickly killed at a lower dose of anti-Fas antibody or more rapidly than the majority of cells. These cells are therefore lost from the treated cultures but are present in the untreated population. Likewise, the plasmids inducing this super-sensitivity are present in the cells before treatment but are absent from the cell sample taken after treatment. Thus, these fragments are identified during the subtraction.

7. The cDNA fragments generated by the subtraction were cloned into the original expression vector. Appropriate restriction enzyme sites were generated or maintained during the subtraction procedure so that the recloned construct is exactly identical to the construct in the originally transfected cells. The sequence of the isolated cDNA fragments was determined.

8. The anti-sense expression plasmids containing the cDNA inserts that were identified in the method of the present invention were individually re-transfected into HeLa cells and the transfectant cells were assayed for sensitivity to anti-Fas antibody treatment.

Specific Materials and Methods

HeLa cells were transfected with anti-sense cDNA library cloned in the episomal vector, anti-sense expression vector pTKO-1. This is the same library described in Deiss and Kimchi [1991]. One million cells plated in a 100 mm dish were transfected with 15  $\mu$ g of DNA containing the anti-sense cDNA library, by using the Superfect reagent (Qiagen, Santa Clarita, California) as suggested by the manufacturer. Two days following transfection, cells were treated with Hygromycin B (200  $\mu$ g/ml) (Calbiochem-Novabiochem Corporation, La Jolla, California). Following two weeks of selection, the population of cells was completely resistant to Hygromycin B.

These cells were plated in triplicate at a density of  $2.5 \times 10^6$  cells per 150 mm dish in the absence of Hygromycin B. One plate was treated with anti-Fas antibody at 10 ng/ml (clone CHI-11 Kamiya Biomedical Company, Seattle, Washington) for five days, the second plate was treated with 100 ng/ml of anti-as antibody for 24 hours and the third plate was UN-treated for 24 hours. Following the treatments, the cells were harvested by washing twice with ice cold PBS (NaCl 8g/liter; KCl 0.2g/liter;  $\text{Na}^2\text{HPO}_4$  1.44 g/liter;  $\text{KH}^2\text{PO}_4$  0.24 g/liter; final pH of solution adjusted to pH 7.4 with HCl) and concentrated by centrifugation (15,000 x g for 15 seconds). DNA was extracted by using solutions P1, P2 and P3 from the Qiagen Plasmid Purification Kit (Qiagen, Santa Clarita, California). The cell pellet was resuspended in 200  $\mu$ l of solution P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100  $\mu$ g/ml RNase A) then mixed with 200  $\mu$ l of solution P2 (200 mM NaOH, 1% SDS) and incubated five minutes at room temperature. 200  $\mu$ l of solution P3 (3.0M Potassium Acetate, pH 5.0) were added and incubated two minutes at room temperature, followed by a ten minute

centrifugation at 15,000 x g. The clear supernatant was mixed with an equal volume of isopropanol and centrifuged at 15,000 x g for ten minutes. The precipitated DNA was resuspended in 100  $\mu$ l of water and stored frozen until use.

For PCT amplification of the cDNA inserts contained in these DNA preparations, the following reaction was set in a total volume of 100  $\mu$ l: 1  $\mu$ l of the DNA, 200  $\mu$ M of dATP, dGTP, dCTP, dTTP, 500 ng each of primers prLPD#64 (SEQ ID No:2) and prLPD#65 (SEQ ID No:3); 10 mM Tris-HCl pH 9.0; 0.1% Triton X-100; 1.0 mM MgCl and 1 unit of Taq DNA polymerase (Gibco/BRL, Gaithersburg, Maryland). This reaction was incubated in a Thermocycler 2400 (Perkin-Elmer, Foster City, California) according to the following protocol: First, the reaction was heated to 94°C for five minutes, then was cycled 25 times using the following three temperatures: 58°C for one minute, 72°C for five minutes, 94°C for one minute. After 25 cycles, the reaction was incubated at 72°C for seven minutes. This resulted in amplification of the cDNA inserts. The prLPD#64 and prLPD#65 primers were design such that the end of the cDNA insert that is proximal to the promoter in the pTKO-1 vector is exactly flanked by a HindIII restriction site (this site is present in the vector) and the end of the cDNA that is distal to the promoter in pTKO-1 vector contains a BamHI restriction site. The BamHI site was created by altering a single base in the sequence immediately adjacent to the distal cDNA insert site (prLPD#65), by PCR. When the library was generated [Deiss and Kimchi, 1991], this site distal to the promoter was generated by the fusion of a BamHI restriction site (derived from the cDNA fragments) and a BgIII site (derived from the vector). This fused site is resistant to cleavage by either enzymes, but a single base change restored the cleavage by BamHI. Thus, the

amplified cDNA fragments are flanked by a HindIII restriction site on the promoter proximal side of the cDNA and by a BamHI site on the promoter distal side. This allows the exact re-cloning of the fragments into the pTKO-1 expression vector with exact conservation of sequence and orientation.

Following the PCR reaction, the mixture was cleaved with BamHI and HindIII (Gibco/BRL, Gaithersburg, Maryland) as described by the manufacturer. The digestion products were purified using the Wizard PCR Prep Kit (Promega, Madison, Wisconsin). This generated cDNA inserts with HindIII and BamHI ends.

These nucleic acid fragments were subjected to subtraction using the PCR-Select Kit (Clontech, Palo Alto, California) according to the instructions of the manufacturer with the following modifications. The driver was the PCR products derived from the untreated samples and two testers were used. The first tester was derived from cells treated with 10 ng/ml anti-Fas antibody and the second tester was derived from cells treated with 100 ng/ml of anti-Fas antibody. First modification: the subtraction is done between dsDNA pools so no cDNA synthesis is required. The fragments generated from the previous step were used directly in the subtraction. Thus, applicants began at Step IV F3 in the instructions (preparation of the adapter ligated tester cDNA). The second modification was the replacement of the blunt end ligation of adapter 1 and adapter 2R with cohesive end adapters. These cohesive end adapters were ligated to the BamHI and HindIII cleaved PCR fragments generated in the step above. The cohesive ligation is usually more efficient than blunt end ligation and since applicants use cDNA flanked by different restriction sites allowing the orientation of the fragments to be maintained when recloning the

subtracted products. If the blunt end ligation is used, it would not allow distinguishing one end from the other and applicants would not be able to determine the relative orientation of the cDNA in the original expression cassette. Thus, adapter 1 was replaced by an equal mixture of primers prLPD#80 (SEQ ID No:4), prLPD#81 (SEQ ID No:5), prLPD#83 (SEQ ID no:7) and prLPD#84 (SEQ ID No:8). Adapter 2R was replaced by an equal mixture of prLDP#82 (SEQ ID No:6), prLPD#88 (SEQ ID No:12), prLPD#89 (SEQ ID No:13) and prLPD#90 (SEQ ID No:14). The other primers were of identical sequence as described in the kit. Thus, primer prLPD#85 (SEQ ID No:9) is the sequence of PCR primer 1, primer prLPD#86 (SEQ ID No:10) is the sequence of nested PCR primer 1 and primer prLPD#87 (SEQ ID No:11) is the sequence of nested PCR primer 2R. The manual supplied by the manufacturer with the kit was followed from the point of ligation of the adapters to the tester (Section IV F3 in the Manual). 0.3  $\mu$ g of the tester was taken for adapter ligation. The initial hybridization included 0.9  $\mu$ g of the driver and 0.03  $\mu$ g of the adapted ligated tester. At the conclusion of the subtraction, a final PCR reaction is done using nested PCR primer 1 (prLPD#86) and nested PCR primer 2R (prLPD#87). This material contains the cDNA fragments that were present in the untreated sample but absent from the treated samples. The product of this PCR reaction were re-cloned into the anti-sense expression vector. (Primers used in this example are set forth in Table 1.)

Re-cloning of the subtracted fragments was accomplished by cleaving the subtracted population with BamHI and HindIII and purifying the cleaved products with the Wizard PCR Prep Kit (Promega Madison, Wisconsin). The cleaved products were then directly cloned into the pTKO1-DHFR vector between the HindIII and BgIII sites. This replaced the DHFR sequences with the cDNA. This is



precisely the procedure that was used to generate the anti-sense cDNA expression library. Thus, the fragments that were generated by the subtraction were exactly re-cloned into the original anti-sense expression vector that was used to transfect cells at the beginning of the procedure. The re-cloned constructs exactly duplicate the constructs that were present in the library. The re-cloned constructs were introduced into bacteria and DNA was extracted from the bacteria following conventional methods. These DNA preparations were used as a template for sequencing in order to determine the nucleotide sequence of the isolated cDNA inserts. Primer prLPD#51 (SEQ ID No:1) was used in Automated sequencing using Applied Biosystems 377XL. DNA sequencer with Perkin-Elmer Dye Terminated Sequencing Kits (Perkin-Elmer, Applied Biosystems Division, Foster City, California). In addition, plasmids carrying the re-cloned inserts were transfected into HeLa cells to confirm their ability to induced super-sensitization to anti-Fas antibody treatment in HeLa cells.

HeLa cells were transfected with 15  $\mu$ g of plasmids or control vectors as described for transfection of the original library. The cells were selected for two weeks for resistance to Hygromycin B treatment (200  $\mu$ g/ml). This selects for cells which contain expression cassettes. One million cells were plated in a 100 mm dish and treated with anti-Fas antibody. Effects of anti-Fas antibody on the transfected cultures were quantified by MTT assays as described by the manufacturer (Sigma, St. Louis, Missouri)

#### ANALYSIS OF THE ISOLATED DNA SEQUENCES

The clones were sequenced using primer prLPD#51 which anneals close to the edge of the cDNA which is distal to the promoter in the antisense expression

cassette. Thus, in the case that the sequence matches the sense strand of a known gene then the insert is in the antisense orientation. Sequences were compared to the combined nonredundant database and the dbest compiled at the NCBI using the Blastn program with default parameters (Internet address: <http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast?Jform=0>). The sequences determined by the method of the present invention are listed in Table 2.

Clone LPD#599 (SEQ ID No:15) shows no match against known gene sequences in the nonredundant database as of November 9, 1997, but does match several EST sequences such as a 99% match against gene bank entry AA043612. Sequence analysis indicates that this fragment is oriented in the sense orientation in the antisense expression library. Applicants have noticed that although the library is designed to preferentially express the antisense strand, there are some sense gene fragments included in the library [Levy-Strumpf et al., 1997].

Clone LPD#601 (SEQ ID No:16) shows no match against known genes in the nonredundant database as of November 7, 1997, but matches many genomic clone pieces and many EST entries as for example a 95% match to a portion of gene bank entry N20920.

Clone LPD#602 (SEQ ID No:17) shows no match against known genes in the nonredundant database as of November 7, 1997. It does show some similarity to a large number of gene bank entries such as gene bank entry Z68269. Many of these matches are in the 60-70% range and may indicate a repeated sequence.

Clone LPD#606 (SEQ ID No:18) shows no match against known genes in the nonredundant database as of November 7, 1997. It does shown some matches against mouse EST in

the 80% range (gene bank entry W71379, W29410 and AA409950) and a stretch of a good match against a human EST (gene bank W19764).

5        Clone LPD#607 (SEQ ID No:19) shows no match against  
known genes in the nonredundant database as of November  
7, 1997. This clone does show a very good match against  
three EST (gene bank entries, T08248, H42827 and T30569).  
The sequence analysis indicates that this fragment was  
10       transcribed in the antisense orientation in the original  
library. Thus, reduction (inactivation) of the gene  
product that is encoded by the full length message  
representing this clone leads to supersensitization of  
cells to the treatment with anti-Fas antibodies.

15       Clones LPD#608 and LPD#618 (SEQ ID No:20, SEQ ID  
No:26) show no match against known genes in the combined  
nonredundant database as of November 7, 1997. They do  
shown a match with a large number of EST entries (for  
example, gene bank entry, AA335297, H14907 and AA009451).  
The sequence analysis indicates that this fragment was  
20       transcribed in the antisense orientation in the original  
library. Thus, reduction of the gene product that is  
encoded by the full length message representing this  
clone leads to supersensitization of cells to the  
treatment with anti-Fas antibodies.

25       Clone LPD#609 (SEQ ID No:21) shows no good match  
against know genes or EST in the combined nonredundant  
database as of November 7, 1997.

30       Clone LPD#610 (SEQ ID No:22) shows no good match  
against known genes in the combined nonredundant database  
as of November 7, 1997, but does show a good match  
against several EST entries including gene bank entries  
AA447349, H24439, R72995, H17221 and R24985. Sequence  
analysis indicates that this fragment was in the sense  
orientation in the original library.

Clone LPD#611 (SEQ ID No:23) shows no good match against known genes in the combined nonredundant database as of November 7, 1997, but does show good matches with a variety of EST (including gene bank entries R76164, R25241, N66591 and N66577). The sequence analysis indicates that this fragment was transcribed in the antisense orientation in the original library. Thus, reduction of the gene product that is encoded by the full length message representing this clone leads to supersensitization of cells to the treatment with anti-Fas antibodies.

Clone LPD#613 (SEQ ID No:24) shows no good match against known genes in the combined nonredundant database as of November 7, 1997, but a portion of the sequence shows homology to a large number of sequences and likely contains a repetitive element.

Clone LPD#616 (SEQ ID No:25) shows an excellent match with human tryptophanyl-tRNA synthetase (see emb X67928 for example). The sequence analysis indicates that this fragment was transcribed in the antisense orientation in the original library. Thus, reduction of tryptophanyl-tRNA synthetase leads to supersensitization of cells to the treatment with anti-Fas antibodies.

Clone LPD#619 (SEQ ID No:27) shows no good match against known genes in the combined nonredundant database as of November 7, 1997, but does show good matches with the sequence of a human retroviral element called pHE.1 (for example emb Z95333, emb Z84475, gb M85205).

Clone L7\_10\_1\_LPD (SEQ ID No:28) shows no good match against known genes in the combined nonredundant database as of November 7, 1997, but does show very good matches against several EST (for example, see gb T77711, gb T78724, gb AA324254). Sequence analysis indicates that the fragment inserted in the expression library was in the sense orientation.

Clone L7\_10\_2\_LPD (SEQ ID No:29) shows no good match against known genes in the combined nonredundant database as of November 7, 1997, but does show good matches with several EST (for example, see gb R12242 and gb H84498).  
5 Sequence analysis indicates that the fragment inserted in the expression library was in the sense orientation.

Clone E7\_100\_11\_LPD (SEQ ID No:30) shows no good match against known genes or EST in the combined nonredundant database as of November 7, 1997.

10 Clone L7\_10\_8\_BS (SEQ ID No:31) shows no good match against known genes or EST in the combined nonredundant database as of November 7, 1997.

Clone L7\_10\_3 (SEQ ID No:32) shows no good match against known genes or EST in the combined nonredundant database as of November 7, 1997.

15 Clone E7\_100\_10 (SEQ ID No:33) shows a good match with mitochondrial DNA (see gb L00016).

Clone L7\_100\_4\_BS and L7\_100\_5\_BS (SEQ ID Nos:34-35) show no good match against known genes in the combined nonredundant database as of November 7, 1997. They do show good matches against several EST entries (for example, see gb AA227149, gb N46661, dbj C76104).  
20 Sequences analysis indicates that this gene fragment was oriented in the sense orientation in the original expression cassette.  
25

Clone E7\_10\_9 (SEQ ID No:36) shows no good match against known genes in the combined nonredundant database as of November 7, 1997. It does show good matches against two EST entries (see gb R54192, gb H39863). The  
30 sequence analysis indicates that this fragment was transcribed in the antisense orientation in the original library. Thus, reduction of this protein leads to supersensitization of cells to the treatment with anti-Fas antibodies.

As described above, the isolated fragments were recloned and then reassayed for sensitivity to treatment with anti-Fas antibody (50 ng/ml for 72 hrs) using the MTT assay. These assays showed that expression of fragments LPD#599 (repeated in triplicate with two different transfectants) or LPD#606 (repeated in triplicate) resulted respectively in a 1.6 and 2.0 fold increase in sensitivity to anti-Fas antibody treatment whereas expression of CrmA (a protective protein) resulted in a 2.3 fold reduction in sensitivity. This demonstrates that the method of the present invention can be successfully used to identify genes based on a positively selected phenotype.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

TABLE 1

SEQ ID No:1	prLPD#51	SEQUENCE: TCTGTAGGTAGTTTGTG
SEQ ID No:2	prLPD#64	SEQUENCE: TGGAGGCCTAGGCTTTTGC
SEQ ID No:3	prLPD#65	SEQUENCE: GTAAGGTTCTTCACAAGGATCC
SEQ ID No:4	prLpd#80	SEQUENCE: CTAATACGACTCACTATAGGGC TCGAGCGGCCGCCCCGGGCAGGTA
SEQ ID No:5	prLPD#81	SEQUENCE: CTAATACGACTCACTATAG GGCTCGAGCGGCCGCCCCGGGCAGGTG
SEQ ID No:6	prLPD#82	SEQUENCE: CTAATACGACTCACTATAGG GCAGCGTGGTCGCGGCCGAGGTG
SEQ ID No:7	prLpd#83	SEQUENCE: AGCTTACCTGCCCCGG
SEQ ID No:8	prLPD#84	SEQUENCE: GATCCACCTGCCCCGG
SEQ ID No:9	prLPD#85	SEQUENCE: CTAATACGACTCACTATAGGGC
SEQ ID No:10	prLpd#86	SEQUENCE: TCGAGCGGCCGCCCCGGGCAGGT
SEQ ID No:11	prLPD#87	SEQUENCE: AGCGTGGTCGCGGCCGAGGT
SEQ ID No:12	prLPD#88	SEQUENCE: AGCTTACCTCGGCCG
SEQ ID No:13	prLPD#89	SEQUENCE: GATCCACCTCGGCCG
SEQ ID No:14	prLPD#90	SEQUENCE: CTAATACGACTCACTAT AGGGCAGCGTGGTCGCGGCCGAGGTA

TABLE 2  
SEQUENCES

SEQ ID No:15 seqLPD#599pr51 (N indicates uncertainty in sequence)

GATCCAAGCGAAAAAGGGAATAAGAAGGGAGGAATGTAAC TAGGAGCAGCTCCCAAC  
AGTTTGCCTATGTATTTGCCAGCACCAAAATTTGTAGAGTAAGCCACTTACATTTCC  
ACTGCTAGTATTAAGGAAAGACAGCAGTGGTGATTTTTATAAAGCGAGTATACATTT  
ATTTTTATTCTGATATGTGAATTTTTCTTTACAGAGTTAATTAAC TGGTAATTTGTA  
AACAGTGGGAAGAAGATTAGAACAATTATGGAGGTACTGAATTACACAAGGAGATTA  
AAATGAAATGAATCAATCTACCTATCTTGTGGTTAGTTAATATTTACCATGATGCAT  
ACACTTGAGAAATGAGAAATGCCCAAATTGTATAATGCATTATCTTNNTTATTATTT  
ATTNNNGTAAATAATTCTTGCTNNACATTANNCTNCAAGGNTAGCTNTATCTATACT  
TGNTAGCTANNTTCTTATACAAAGCANGNTNCTTTTGAANATGATTTACCNATTAAN  
TANANNAGCTTAGGTGCCTNNTTTNACTCTGGNTNGTCATNTTGNTTNNCTTTTNNC  
NANATCATATATAATTTCCANNGAATGTTGATNTNTNTCCTCANNTTNCATNTTANC  
CNGNGCNTATCCTNTNNNTGNTNGNNTATGTC

SEQ ID No:16 seqLPD#601pr51

GATCCTGGTATAATGTGATTGAATTATGAAGGCAAATTTCTCATGAATGGTTCAGCA  
CCATCCCCTTGTACCATCCTCACAATAATGAGTAACTTCTCATGAGATGTAGTCACT  
GAAATCTCTATATCACCTCCCCACTCTCCGTGTTTTCTCCTTGCCATGTGAGACAAT  
TGATTCTTTCTTTGCCTTCCATGATTATTGAAAGATTTCTGAGGCCTAGAAGCAGAA  
GCACTGTGCTTAGAACCATGAGCCAATTAAACCTCTTTTTCAAATAAATCATACGG  
GAAATGGCAAATGAGGACTGGAGCGTTGCTATAAAGATACCTGGAAATGTGGAAGCA  
GCTTTGGAACCAGGTAATGGACGGAGGTTGGAAGAGTTTGGAGGGCTCAAAGAAGA  
CAGATGAGAAAAC TTTTGGACCATCTTAGAGTCTGGTTCAATGGTTGTGACAAAAT  
CCTGACAGAAACATGGACAGTGAAGGCCAGGCTGAGGAGGTCTCAGAGAGAAATAAG  
AAGCTTTTTGCAA

SEQ ID No:17 seqLPD#602pr51

AAACATTATGCACAGAGGAATAAAATTAAGAATGACCACAGTCTTCTCTTCAGAAAT  
TATGCAAGCCAGAATAGAGTAGAGCAACATCTTTACAGTTCTTAAAGAAAAAATATA  
TCAACTTAGAATTCTATACCCAGCAAAAATATATTTCAAAAAGAAGACACAATACTT  
TTTCAGACAGACAAAAGCCAAGAGAGTAATTCAGGGACGTGTAATATAAGAAATAT  
TAGTGAAAGTAGTGTAAGAATGAGAGAGAGGAAATATAAGGATACTGTTATAATAT  
CCCTACACTGTACCTTAAGTGGATTGTAATATTATCTGAAGGTAACTGTAATATGT  
TAAATAAAGATGTATATTTTTAATCCTAGAGAACTGCTATGAAAACAAAAGTAAA



ACAAAGTGGTATTACTAACAAGCCAATAATGGAGATAAAATGGGCAATAGAAAACAA  
CAACAAAAATTCAATCCAAAAGAAGGAAG

SEQ ID No:18      seqLPD#606pr51

GATCCCTCTGGCACTGGGATGCTTCCACAGATGTACCTGAAAAGTCGTCACTCTCTC  
ACCAAGGAGGAAGAAGCAGTACTTTTTTGTCTCATAGCATTAGTAACCAAGCTAATG  
TTCACCAGTCTGTCATTAGCTCCTGGCTCAGCACTGATCCTGCAAAGACCGAATTG  
AAATCACAAGCT

SEQ ID No:19      seqLPD#607pr51

GGGACATGGTTGGGCCATGCCACACCAGGGCTGGTGAGGCAACCAGTTTTGATTTTG  
ACAGAGTGGCTGGAGGAAAAGTGGCAATCAAGGTGCTGCTTGGTTTGCTCTGAGTGC  
AAATGGAACCAACAGGTTTCTGCTGCAATCTGTGTGTTCCCAGTGCCAGGTCACACC  
AGGAGGGGTGGGGCAGGGCTAACCAAGTGGTCTCTGAACTCACCGAGCGTCTGCACT  
TGGTTGTGAAGTTAATGGGAGTACAGAGAGCGTCTGGCCTTGGAGAGGGGTTGAGAG  
CCTCCTTTTTGGTTCTTCATTCCTGAGCTCTTGCCTGCCCACAAATCTGACCTCTTT  
GAATGGGGACGCAGTCCTTCAACAGAGAAGTTTCTATGGCAAAGAAGTTTCTATTTA  
GCTCTAGATCCAGCAGAGTCATCCATTCTAACTGCCCTGAAGTCTAGAGCAGGGGAG  
GGAACCCAGAGGCTGGGGATGAGACTAGGCAGACCCTGGTTACCATATGGACAAGGA  
CAGGGGAAAGCACCCCTTCCCTCAATTTCTGAAAGTTCTATCTTTGGGTTGCTGGA  
CTTTGAGGATGATAAAGAACATNTAGGTACTA

SEQ ID No:20      seqLPD#608pr51

AAGTGGGACTCTGGGCCTCTGACCAGCTGTGCGGCATGGGCTAAGTCACTCTGCCCT  
TCGGAGCCTCTGGAAGCT

SEQ ID No:21      seqLPD#609pr51

GATCCTANACCTGCTCTTTCTCTAATCTTCTGTATCTTAGCAATCGGCCAAAAA  
TGAAATCATACTTGATTTCTCCTGGCCCTCAAAGCCTACATTCATTCTGCCAAATCA  
TGTAAGTCCATCTTTAAAATTTATACTGACTGTGCATTTCTTCTTATCTCCATTACT  
ATCACCCAAGTTCAAATCCTTTCTTATTTTTTACCTGAGTATTGCAATATTTTCTT  
AGATGACCCTAACTGATCTTGCCGTTTCTACTCTTGCTTTCCTACAGTCAATTCTCT  
TCATATATGCCAACATTACCTTTTAAAACCTACAAACAAGTTCAGGTTACTTCTTCCT  
TGCTCAAAGTCTCAAATATCTTTTTATCACACACAAATCAAAGGTGGCTAAGAATGG  
TCTGGGTCTGCATACCTCTCCAATGTCATCTACAGCATCTCTGACTTTCTCGCACT  
GCTCTGGTCACAGTGGTCTTCTTTTTCTCTAATACACTAAG

SEQ ID No:22      seqLPD#610pr51

TCCAGAGCAAACATTACATCATAGGCCATGGGGGTGTCACTTAGCTGCATGCTAAG  
AATCCTCATATGCTGCTTTGAAAACGCCATAAAAACTAAGATGCCCTCATACACAG

AAAAAAGGAGTTGGGCCAACAGCCTGCGAGAGAAGATCCTTCCCAACTCTGCCATTCT  
TTACTGCAGGAGGGTTTGTAAATGCTTCCCCACTTCCTCAGGTCCAGCGACTGCACCT  
CCACCACTGGAGTGGGGGACACTTCCATTGCTGATGGAAGCTGCCTACTGCTTTTAA  
AAACACACACATGAGCTGAGAAGTTCTGCTGAAGGTGGGGAGAGTGCTGCTCTTGGC  
TGCCAGCCGGGTCTAGCGGCCCTAACAAAGGGAACTGGCTGATACAAAATGGATCAC  
TGCCTGAGATATATGAACTGGACTGAGGATAGAAGTTGCATCCGCTGGGCAAAATGT  
GCCAAAGAACAGAAAGTCTTTCAATTTATTCTTTTAGGCACCAAGAATAAATAAATN  
CTAAGA

**SEQ ID No:23      seqLPD#611pr51**

GGGTGAAGCATCTGNTTCATNACACAGGGCTATCATAATTGGGTCTTGAAATGGAA  
TGGGTCTCTAAACATTTTAAATGCCCCAGCTTGGTATTGGACNCAATGCAAGATGGCT  
AAAATCTTCATTTAAAATTCAAGGGTATTGCTTGCTATTTACATTATATACTTTTT  
AAATACTATTGCTCTTTGCATATGAGGCATTTCACTAACCTTGGATCATTGGATCTT  
AACATGATAATATAGAATATCTGAACATTGGACTTGNTCTTTAGAGTATATGGCCT  
TTAGTTCTTGTGGAATAAATACAGTGATTCTAAGATCAAAAGTCTTAGTGTTTGGG  
GTTTTTTTTTCTCTTTTGGATATGGGGTGTT

**SEQ ID No:24      seqLPD#613pr51**

ATCCAGCGGNTCAGCACACACTGGGGCCTACCGGAGTGGGAGGGAGGGACAGGGATG  
AGGGAAAAATAACTAATGGGTACTAGGCTTAATACCTGGGTGGTGAAATAATCTGTA  
CAACAACTCCCATGACACAAGTTTACCTTTACAAACCTGCACATGTACCCCTGAAC  
TTAAAGAAAAGTTTTTTTAAAAAATCACACTTCCAGAGTTTGCTAAATAATTATGAC  
CAGTATTTTACTACCATCTTCTTCTTGGTGAATACTAAATTAGTAAATTTATGT  
TAAAAATGAAGTTCTCCCTAGAAATCAAAAGTGCAGTCTAAGCACTGAAAATGTTCT  
ATCAACACTTGTTAACTGAGAACCATTGAAACATCTAGGA

**SEQ ID No:25      seqLPD#616pr51**

TNCGGATGAGATAGTGAAAGAGTTCATGACTCCCCGGAAGCTGTCCTTCGGCTTTCA  
GTAGCACTCGTTTTACATATGCTTATAAAAGAAGTGATGTATCAGTAATGTATCAAT  
AATCCCAGCCCAGTCAAAGCACCGCCACCTGTAGGCTTCTGTCTCATGGTAATTACT  
GGGCCTGGCCCCCTGTAAGCCTGTGTATGTTATCAATACTGTTTCTACCTGTGAGTTC  
CATTATTTCTATCTCTTATGGGCAAAGCATTGTGGGTAATTGGTGCTGGCTAACATT  
GCATGGTCCGGATAGAGAAGTCCAGCTGTGAGTCTCTCCCCAAAGCAGCCCCACAGT  
GGAGCCTTTGGCTGGAAGTCCATGGGCCACCCTGTTCTTGTCCATGGAGGACTCCGA  
GGGTTCCAAGTATACTCTTAAGACCCACTCTGTTTAAAAATATATATTCTATGTATG  
CGTATATGGAATTGGAAATGTCATTATTGGAACCTAGAAANGGCTTTGGAATATTGA

TGTGGGGAGGNTTATTGAGCACCAGATGTATTTTANCCCATGCCCCCTCCAAAAAGA  
AATGGTTAGTNAAAAC

SEQ ID No:26 seqLPD#618pr51

GATCCTTNCAAGTGGGACTCTGNCCTCTGACCAGCTGTGCGGCATGGGCTAAGTCA  
CTCTGCCCTTCGGAGCCTCTGGAAGCT

SEQ ID No:27 seqLPD#619pr51

CTNTGGANTACANCNATCCCTGGATTTAANGAGANGGCCAGGCCACTCTATACTCTA  
ATCAAGGAGACCCAGAGGGCAAATACTCATCTAGGAGAATGGGAACCAGAGGCAGAA  
ACAGCCTTCAAAACCTTAAA

SEQ ID No:28 L7\_10\_1\_LPD

AATAAGGTTCCCTTCACAAANATCCAAGCCAGCACCTTAGTTTTCTACAACATAAAT  
GTAACAAAGTTATCTTCTACTGTATTGCACCTTAGTCCAAAAGTAAACAACATAAAT  
GAAAATTTAAATAAATCAGACTGAAAAAGCCCCAAAGAGTAAGAGGAATACCTTATAA  
ATGTGACTACCCACCTAAAAATTCTTGAACACTTTGTTTTGCATAGGATTTTATGG  
GACTAACCAAATGTTCCATGAACCATGAGGTGAAAACTGCGATTTTCATGATAGCACA  
TTGTTTTACAATTCTGATTAGAAATCCTTCAGAAATATTTCTGTATC

SEQ ID No:29 L7\_10\_2\_LPD

ANGTAAGGTTCCCTTCACAAAACCAAGCGGGACTCCNAAACAACCATGGTGCAATGG  
TGGCAAAAACCTGGGGACTGGGTCTTTCTCGCAACTTCTGTGCCTCCTTTCATTCTCA  
AAAAAGGACTATTACCAAATGGGGGGGAAAAAACATAAGCANAAAAAACCCCAAGGA  
CAAACCTGGAAATTTAATTCCTTNATGCAATCTTATCTTCATCTGGTGCCTCATTAC  
CCTGGGCCCCAAGCCTTCTAATTAGGAAAAAATACTGATTTCTGTTAGGCAATTGCT  
TATNTTGGTGGCTTCATCACATTTCA

SEQ ID No:30 E7\_100\_11\_LPD

CAAAGTACCAAGCCCGAGAAAAATACAGGTCTATTCAATTCCACCTGCTAGCCTAAAC  
TTCAAGTAGACTTCAAAGCAATACAAGTTTGTATGGTCTTTTTGGCAATAGCAGGAA  
ATGATACAAGCGAAATCAGTTCTTGATTAAGGGAGGAGCAGAATTGCATTAGTTAGA  
TATTCTGGTAGTGCTGCAAACTATAATGATAAATGAAGGCAGCCCAAGACTTAAGAT  
GTAAAGTTATGTAGCTCATGCAATTCAATGTCAGTATTTGAAGGCTATATGATGAAT  
ATTTCCAGAATTATAATGAAAAAGTAAAAACAAATTTGCCTCTTACTGATGCTTCA  
AAAATCATTGTGTATATTTAACAAAAGAAGTGTCTAAATAGATCTGAATTTAAACC  
ACAGTACTGAACACACTACATGAGGTAACATTGAGTATTATCAAAGTACCAAGCCCCG  
AGAAAATACAGGTCTATTCAATTCACCTGCTAGCCTAAACTTCAAGTAGACTTCAA  
AGCAATACAAGTTTGTATGGTCTTTTTGGCAATAGCAGGAAATGATACAAGCGAAAT  
CAGTTCTTGATTAAGGGAGGAGCAGAATTGCATTAGTTAGATATTCTGGTAGTGCTG

CAAACCTATAATGATAAATGAAGGCAGCCCAAGACTTAAGATGTAAAGTTATGTAGCT  
CATGCAATTC AATGTCAGTATTTGAAGGCTATATGATGAATATTTCCAGAATTATAA  
TGAAAAAAGTAAAAACAAATTTGCCTCTTACTGATGCTTCAAAAATCATTGTGTAT  
ATTTAACAAAAGAAGTGTCTAAATAGATCTGAATTTAAACCACAGTACTGAACACAC  
TACATGAGGTAACATTGAGTATTAT

SEQ ID No:31 L7\_10\_8\_BS

AAGCAAAAGGAATGCTTATACACTGTTGGTAGGAGTATTAGGATTACAGGCGTGAGC  
CTCCACTTCCAGCCGTCCACAATCTATTGAGATGACCATGTTGTTTTCTTCCTTCAG  
TCTGTTGCACTAATCATTTCAGCTGATGAAATAACGTTGCATTCCAGGGATACACT  
GTACTGGGTACTGGTGTACATTCCTGTTTATATGTTACGGTATTTGGTTTGCCAAAA  
TTTACTAGGAAATTTACAGATCCACTCATAAGCGATATTATCAGAGTTTTCCCTGT  
GTATGATGTTTTCACTTTGGTATCTAGATAACAATAGCATCATAGAACCAGTAAAAAA  
TATTACATGCTCTTCCAAGTTTTTAAGACATTTTTATCGAATTGGTAATTTTCATGT  
GTTTGGTATAATATACAAAAGAATACATTTGAACTGGGAATTTACATGTGTGTCAGG  
GGCAGGGGTGAGAGTTTTTAAATTACCAATTTATTGTCTTCACTTTGTTCTTGAATG  
AGTTGTGGTAGTCAGTGTCTTTCTAGGAATTTGTCTACTTCCACAAAGATATTTGTC  
AAAATACTAATCCCTTGTTATGGGAA

SEQ ID No:32 L7\_10\_3

AAGCGGCACTCATCACTTCTACCCCTTATTTCTGCTGGCAGAAACCCAGTCACAAGCT  
CCACTGACATGCAAGGAGATTTGGGAAATGCAGTCTCTGTGCTAGCCCAAACCTCT  
AAGAAGCCAAAGGAAATATGTATTTTGGGTGGAGATCTAGCCATCTTACCACACTAT  
GGTGGTCCAGTAGAGGTCTATCAAAATATTAATTCACAGAAGACTAAAGACACATTT  
ACAATGAAGGTTTACAAAACCTTATCTGCAAAGAAAAAGCCAGAACATGTTTTATTGT  
GGAATAGTTCTAAAATTGCTTATAGATGAAAAGAACAAAACAAATATTTAAATCAGT  
CACCTCTAGAATAGTGAAAGGCCAAAACCTGCATTTTCAGAAATGAAATATCACTCTG  
GGA

SEQ ID No:33 E7\_100\_10

AAGCGAAAACACCCTCATGTGAATGAGGGTTTTATGTTGTTAATGTGGTGGGTGAGT  
GAGCCCCATTGTGTTGTGGTAAATATGTAGAGGGAGTATAGGGCTGTGACTAGTATG  
TTGAGTCCTGTAAGTAGGAGAGTGATATTTGATCAGGAGAACGTGGTTACTAGCACA  
GAGAGTTCTCCAGTAGGTTAATAGTGGGGGGTAAGGCGAGGTTAGCGAGGCTTGCT  
AGAAGTCATCAAAAAGCTATTAGTGGGAGTAGAGTTTGAAGCCCTTGAGAGAGGATT  
ATGATGCGACTGTGAGTGCGTTTCGTAGTTTGAGTTTGCTAGGCAGAATAGTAATGAG  
GATGTAAGTCCGTGGGCGATTATGAGAATGACTGCGCCGGTG

SEQ ID No:34 L7\_100\_4\_BS

TACTGTGATGTTGTTGCTATCTTCATCATCTAACACCTGTGATTTTATATCCATGGT  
CACATATGGAAAACCCCAAGGACAGCCATAACCTCTTCATATTTTTCATCTTCAAG  
GAAGTGCAGTAGAGTGTGACGATCTGATTCTTTTAAACTCACCAAATCCTGGATAGT  
TTTAATTTTATACTTCTTATGATTAGAAACCCGTCTAAGATTGTCCTCTTCAATATG  
AGGGAGCTGCAGAAGGGGAGACTTAAATTGCTGAAGTCCCTGAACGGCCATCTGAGA

SEQ ID No:35 L7\_100\_5\_BS

TACTGTGATGTTGTTGCTATCTTCATCATCTAACACCTGTGATTTTATATCCATGGT  
CACATATGGAAAACCCCAAGGACAGCCATAACCTCTTCATATTTTTCATCTTCAAG  
GAAGTGCAGTAGAGTGTGACGATCTGATTCTTTTAAACTCACCAAATCCTGGATAGT  
TTTAATTTTATACTTCTTATGATTAGAAACCCGTCTAAGATTGTCCTCTTCAATATG  
AGGGAGCTGCAGAAGGGGAGACTTAAATTGCTGAAGTCCCTGAACGGCCATCTGAGA

SEQ ID No:36 E7\_10\_9

AAGCGGACTTTGGAGGGCAGTGTATTTTCCCAAAGAAAGACGGCCAAGGGCAGAGG  
CATGGATTCTTTGCAGAGCACTTCCTTTTGGTTTTTCAGTACTGTTTCATAGACAGT  
GGGCTCACATGTTCTGATAGTGCTGCAGTTGCTTAGAAAGCATCCCAGTTAATTGC  
AGTAATTAGAACTTCTGGAATATGCTAGGGCAGAAGTATGTCAAGTATGTCACATGA  
AGAAAATGTGAAATTCAAGAGTAATCCACACGTGAGAACTAGACAATGTACATTCA  
TGTGTTCTCTTGAAAGGAAAGGGAGAGCTGTAAGCTATCGATACCGTC

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CLAIMS

What is claimed is:

1. A method for the identification of genes that are essential for the maintenance of specific cell phenotypes including the steps of:

- a) identifying a cell type with a phenotype of interest;
- b) inactivating genes in the cell type of interest with a gene inactivation means on an aliquot of a culture of the cell type;
- c) applying positive selection means to an aliquot of the cell culture of step b;
- d) observing cells which continue to maintain the phenotype following gene inactivation whereas cells in which genes necessary for maintaining the phenotype have been lost; and
- d) utilizing subtraction analysis means to identify the gene in the cells which has been inactivated that affects the phenotype of interest.

2. The method as set forth in claim 1 wherein said gene inactivation means are performed using a Genetic Suppressor Element (GSE) inactivation, a Random Homozygous Knock-Out (RHKO) inactivation, or a Technical Knock Out (TKO) inactivation.

3. The method as set forth in claim 1 wherein the phenotype of interest can be selected from the group consisting of phenotypes relating to growth, phenotypes relating to release of factors and phenotypes relating to cell functions.

4. The method as set forth in claim 1 wherein the positive selection means can be selected from the group

consisting of ability of cells to survive under specific culture conditions, ability to express a specific factor, changes in cell structure, and differential gene expression.

5. The method as set forth in claim 1 wherein the subtraction analysis means are selected among the methods of differential display, representational differential analysis (RDA), suppressive subtraction hybridization (SSH), serial analysis of gene expression (SAGE), gene expression microarray (GEM), nucleic acid chip technology, or direct sequencing.

6. A gene identified by the method of claim 1.

7. An antibody raised against a gene product whose sequence is set forth in the gene set forth in claim 6.

8. A method for the identification of genes that are essential for the maintenance of specific cell phenotypes including the steps of:

a) identifying a cell type with a phenotype of interest;

b) preparing an expression cDNA library constructed for the cells as they are expressing the phenotype;

c) transfecting a cell culture of the cell type with an anti-sense expression vectors incorporating the expression cDNA library;

d) applying positive selection means to an aliquot of the transfected cell culture and reserving an untreated aliquot;

e) observing cells which continue to maintain the phenotype and isolating the antisense expression vector from the cells maintaining the phenotype;

f) identifying anti-sense expression vectors that are present in the reserved aliquot and not in cells maintaining the phenotype by subtraction means whereby anti-sense expression vectors are identified that have targeted genes that maintain the phenotype.

9. The method as set forth in claim 8 wherein the step of recloning and sequencing the antisense expression vectors that target the genes that maintain the phenotype is performed on the identified antisense expression vectors.

10. A kit customized to provide reagents to perform the method of claim 8 including  
a culture of cells of the requested phenotype;  
and  
an expression cDNA library constructed for the cells as they are expressing the phenotype.

11. A nucleic acid having a sequence such that an antisense construct of the sequence inserted into a cell provides reduction of a gene product and thereby sensitization of the cells to anti-Fas antibody.

12. The nucleic acid as set forth in claim 11 having the sequences set forth in SEQ ID Nos:19,20,23,25,26,36.

13. The nucleic acid as set forth in claim 11 wherein the cells are HeLa cells.

14. A nucleic acid having a sequence as set forth in SEQ ID No:18.

15. A nucleic acid having a sequence as set forth in SEQ ID No:21.

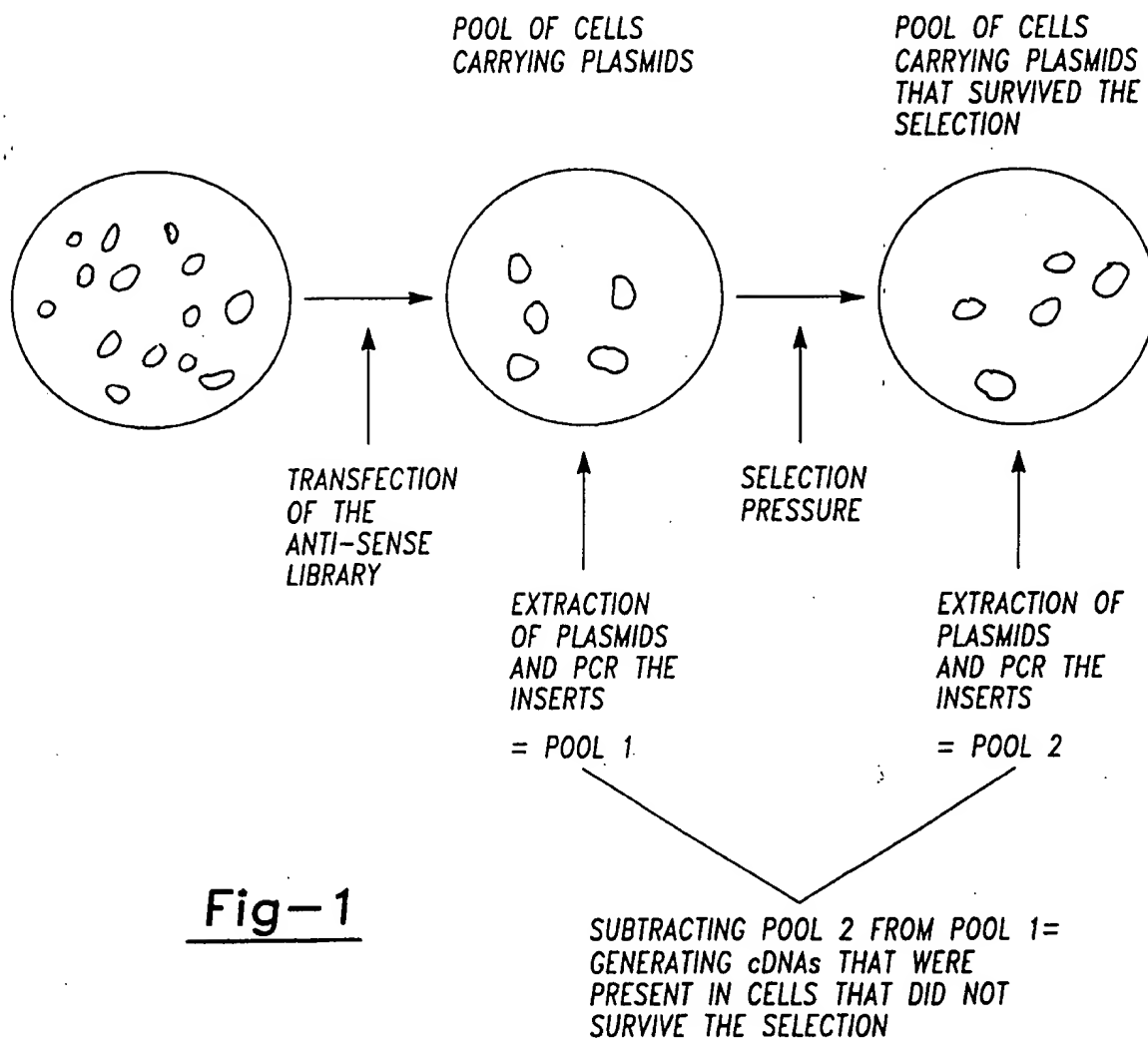
16. A nucleic acid having a sequence as set forth in SEQ ID No:25.

17. A nucleic acid having a sequence as set forth in SEQ ID No:30.

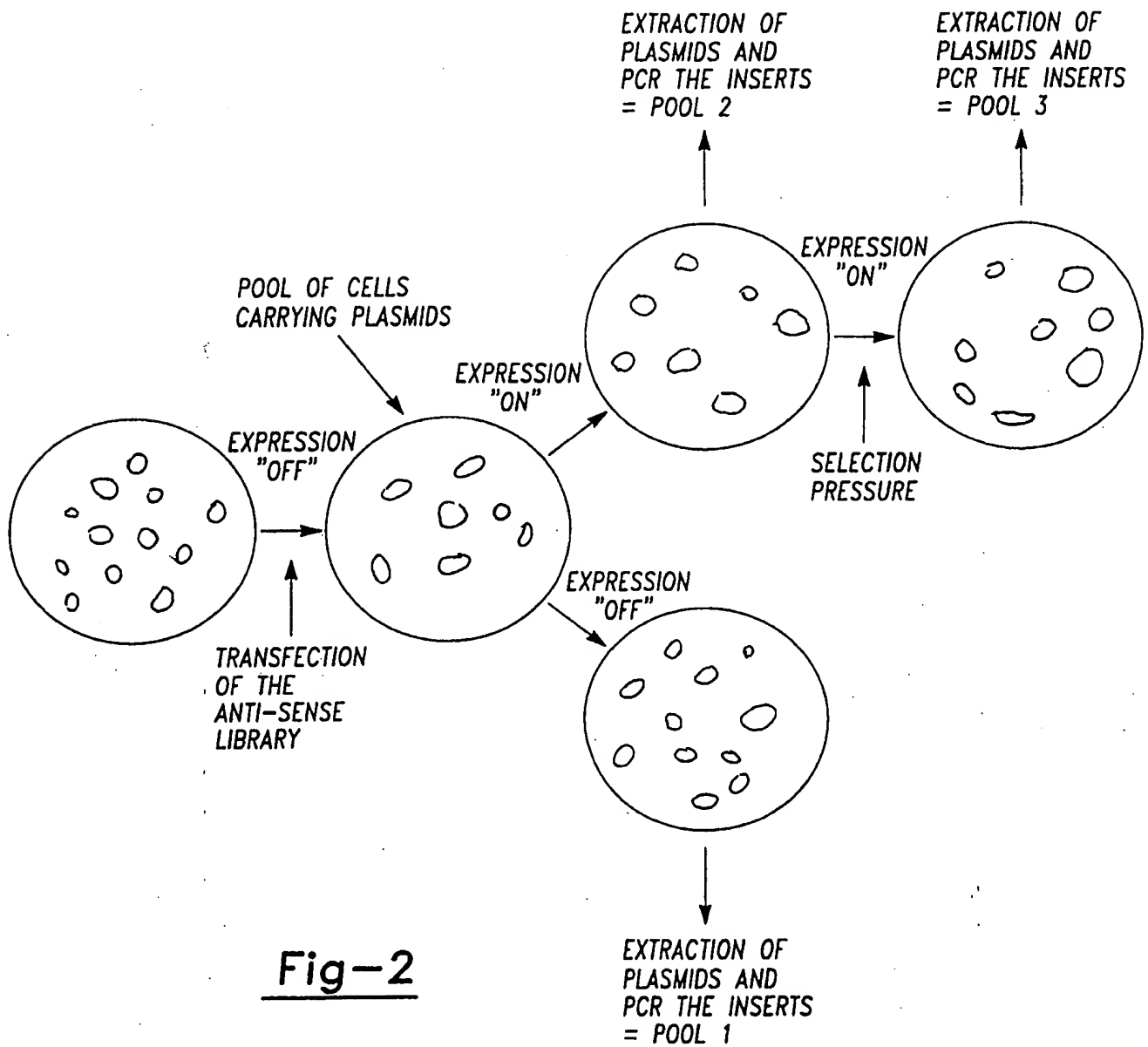
18. A nucleic acid having a sequence as set forth in SEQ ID No:31.

19. A nucleic acid having a sequence as set forth in SEQ ID No:32.

1/2



2/2



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20989

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68, 1/02; C07H 21/04

US CL : 435/6, 29; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 29; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS (USPAT, EPOABS, JPOABS), Medline, Biosis  
search terms: subtraction, library, antisense

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Lisitsyn et al. Cloning the Differences Between Two Complex Genomes. Science. 12 February 1993, Vol. 259, pages 946-951, especially page 946.	5
X --- Y	Deiss et al. Identification of a novel serine/threonine kinase and a novel 15-kd protein as potential mediators of the gamma interferon-induced cell death. Genes & Development. 1995, Vol. 9, pages 15-30, especially page 15.	10 ---- 1-5, 8-9
Y,P	US 5,604,118 (GIRI ET AL.) 18 February 1997, column 7, lines 46-52.	1-5, 8-9



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 JANUARY 1998

Date of mailing of the international search report

13 MAR 1998

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/20989

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Deiss et al. A Genetic Tool Used to Identify Thioredoxin as a Mediator of a Growth Inhibitory Signal. Science. 05 April 1991, Vol. 252, pages 117-120, especially page 117.	10 ---- 1-5, 8-9
Y	Klickstein. 'Production of a Subtracted cDNA Library.' Unit 5.8B In: Current Protocols in Molecular Biology. Edited by Ausubel et al. John Wiley & Sons, Inc. 1992, pages 5.8.9-5.8.15, especially pages 5.8.14-5.8.15.	1-5, 8-9



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20989

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-5, 8-10

Remark on Protest

☐  
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-5, and 8-10, drawn to a method of identification of genes and a kit used in the method.

Group II, claim 7, drawn to an antibody specific for a gene isolated by the method of Group I.

Group III, claim(s) 6, and 11-19, drawn to genes.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature is a cell screening method comprising a subtraction analysis. The antibodies of Group II are not used in or a product of the method of Group I and therefore lack the special technical feature of Group I. The genes of Group III are not a product of the method of Group I, which is a screening method, and therefore lack the special technical feature of Group I.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

Species I consists of SEQ ID Nos: 19, 20, 23, 25, 26, 36, 18, 21, 25, and 30. Species II consists of SEQ ID Nos: 31 and 32.

The claims are deemed to correspond to the species listed above in the following manner:

Claims drawn to species I are: 12, 14, 15, 16, and 17. Claims drawn to species II are: 18 and 19.

The following claims are generic: 6, 11, and 13.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Species I and II lack a corresponding special feature because: They are materially different products lacking common sequences representing a common special technical feature. Therefore the species are not so linked as to form a single inventive concept under PCT Rule 13.1.